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Characterisation of thapsigargin-releasable Ca^{2+} from the Ca^{2+}-ATPase of sarcoplasmic reticulum at limiting [Ca^{2+}]

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Abstract

The Ca^{2+} binding sites of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum (SR) have been identified as two high-affinity sites orientated towards the cytoplasm, two sites of low affinity facing the lumen, and a transient occluded species that is isolated from both membrane surfaces. Binding and release studies, using $^{45}Ca^{2+}$, have invoked models with sequential binding and release from high- and low-affinity sites in a channel-like structure. We have characterised turnover conditions in isolated SR vesicles with oxalate in a Ca^{2+}-limited state, $[Ca^{2}]_{lim}$, where both high- and low-affinity sites are vacant in the absence of chelators (Biochim. Biophys. Acta 1418 (1999) 48–60). Thapsigargin (TG), a high-affinity specific inhibitor of the Ca^{2+}-ATPase, released a fraction of total Ca^{2+} at $[Ca^{2+}]_{lim}$ that accumulated during active transport. Maximal Ca^{2+} release was at 2:1 TG/ATPase. Ionophore, A23187, and Triton X-100 released the rest of Ca^{2+} resistant to TG. The amount of Ca^{2+} released depended on the incubation time at $[Ca^{2+}]_{lim}$, being 3.0 nmol/mg at 20 s and 0.42 nmol/mg at 1000 s. Rate constants for release declined from 0.13 to 0.03 s^{-1}. The rapidly released early fraction declined with time and $k = 0.13$ min^{-1}. Release was not due to reversal of the pump cycle since ADP had no effect; neither was release impaired with substrates acetyl phosphate or GTP. A phase of reuptake of Ca^{2+} followed release, being greater with shorter delay (up to 200 s) following active transport. Reuptake was minimal with GTP, with delays more than 300 s, and was abolished by vanadate and at higher [TG], >5 μM. Ruthenium red had no effect on efflux, indicating that ryanodine-sensitive efflux channels in terminal cisternal membranes are not involved in the Ca^{2+} release mechanism. It is concluded that the Ca^{2+} released by TG is from the occluded Ca^{2+} fraction. The Ca^{2+} occlusion sites appear to be independent of both high-affinity cytoplasmic and low-affinity lumenal sites, supporting a multisite 'in line' sequential binding mechanism for Ca^{2+} transport.

Keywords: Sarcoplasmic reticulum; Ca^{2+} transport; Ca^{2+}-ATPase; Thapsigargin; Ca^{2+} occlusion; Channel

Abbreviations: AcP, acetyl phosphate; $[Ca^{2+}]_{lim}$, limiting concentration of medium or cytosolic free calcium ions; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*,*N*′,*N*′tetraacetic acid; E-P, phosphorylated forms of the Ca^{2+}-ATPase; E_1-P, forms with high-affinity cytosolic-orientated Ca^{2+} binding sites; E_2-P, forms with low-affinity inward-orientated Ca^{2+} binding sites; E_2-P⟨Ca_2⟩, phosphoenzyme with occluded Ca^{2+}; E_2^A.TG, non-phosphorylatable stable isomer of E_2.TG; F_{min} and F_{max}, fluorescence of Fluo-3 with excess EGTA, and with saturating [Ca^{2+}]; MOPS, 3-(*N*-morpholino)propane sulfonic acid; SERCA, sarco- and endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; TG, thapsigargin

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1. Introduction

The P-type cation pumps include the plasma membrane Na^+,K^+-ATPase, Ca^{2+}-ATPase of sarco- and endoplasmic reticulum (SERCA), the plasma membrane Ca^{2+}-ATPase and gastric H^+,K^+-ATPase. All of these systems share a remarkable number of characteristics, including topographical features, conserved residues, phosphoenzyme intermediate species (EP), catalytic cycles, and presumably also similar energy transduction mechanisms (for review, see [1]). Primary transported cations, Na^+, Ca^{2+}, and H^+ bind with high affinity to the E_1 species, and become occluded before transition to the low-affinity E_2 conformation, prior to their release to the *trans* membrane surface. There is general agreement as to the functional properties of these three states, however it is still uncertain whether the sites represent transient alternate conformations of the pump protein, or whether they exist permanently, and that transported cations migrate sequentially from one site to another [2].

Two Ca^{2+} ions bind with high affinity and positive cooperativity to cytoplasmic oriented sites on E_1 [3]. Binding of the first Ca^{2+} (site I) augments binding to the second site (site II), which in turn 'locks' the first deeper site, such that it is relatively stable in EGTA or to displacement of $^{45}Ca^{2+}$-labelled in the first site by $^{40}Ca^{2+}$ in the medium [4,5]. These findings lead to a model in which $E_1.2Ca^{2+}$ binding sites are located in a shallow well or channel-like structure [6]. Properties of E_2-type low-affinity lumenally orientated binding sites are less certain. Initially it was reported that these low-affinity sites did not show first-in first-out behaviour. There is now convincing evidence for four Ca^{2+} binding sites [7–11]. Failure to observe first-in first-out behaviour has been explained by scrambling during the occluded state [12].

Site-directed mutagenesis has provided understanding as to the nature of the Ca^{2+} ligands on putative membrane helices, M4, M5, M6 and M8 (see [2] for review). These include Glu^{309} in M4, Glu^{771} in M5, Asn^{796}, Thr^{799}, and Asn^{800} in M6, and Glu^{908} in M8. Mutations to Glu^{309} and Asn^{796} appear to decrease binding to site II, whilst mutants E771Q, T799A and E908A are involved in Ca^{2+} binding to site I. Ligands between M4 and M6 may be assigned to site II, while those between M5 and M6 can be assigned to site I. In order to account for ligand assignment that cannot be explained by the in-line stacking model, MacLennan et al. have proposed a 'side-by-side' structure in which the pathway of Ca^{2+} translocation would be angular, rather than more direct [13].

The sesquiterpene, thapsigargin (TG), is a specific and potent inhibitor of sarco- and endoplasmic reticulum Ca-ATPases (SERCA), and is used to increase cytosolic [Ca^{2+}] in a wide range of tissues [14–16]. Although TG binds to E1 and E2, it favours E_2, and is competitive with Ca^{2+} because it promotes the reaction $E_2.TG \rightarrow E_2^A.TG$, a stable form of E_2 that cannot be phosphorylated by P_i [17]. Since TG inhibits Ca^{2+} uptake into intracellular stores, it is generally believed that the mechanism by which it increases cytoplasmic [Ca^{2+}] is to allow continued leakage that is not balanced by active uptake in a pump/leak system [18–21].

In recent studies in our laboratory, we have characterised the properties of the Ca^{2+}-ATPase when both high- and low-affinity Ca^{2+} binding sites are unoccupied as a result of transport into SR vesicles in the presence of oxalate [22]. Since no high-affinity chelators are used to limit medium $[Ca^{2+}]_{free}$, small changes in calcium release or uptake can be monitored by a Ca^{2+} fluorophore, Fluo-3. We now report on the TG-induced release of Ca^{2+} from sites on the Ca^{2+}-ATPase under conditions in which both high (E_1) and low (E_2) affinity Ca^{2+} binding sites are vacant. Demonstration of Ca^{2+} occlusion sites, independent from cytoplasmic and lumenally oriented sites, suggest a multisite model for active Ca^{2+} transport.

2. Experimental procedures

2.1. Materials

The sources of materials were as follows: ATP, Sigma; amylase, Boehringer Mannheim; Fluo-3, pentammonium salt, Molecular Probes (Eugene, OR); thapsigargin, lot 12841453, was obtained from Sigma. Stock solutions, 1.0 mM, were prepared in DMSO and kept at −10°C. Standardised 100 mM $CaCl_2$ solution was prepared from Analar $CaCO_3$, adjusted to pH 5.6 with 1 M HCl.

2.2. Preparation of skeletal muscle sarcoplasmic reticulum vesicles

Isolated sarcoplasmic reticulum vesicles were prepared from the back and hind leg muscle of white rabbits by the method of Champeil et al. [23]. Amylase, 1 mg, was added to the initial 460 g homogenate in order to decrease glycogen content and phosphorylase contamination to less than 5%, as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate [24]. Protein concentrations were determined from the optical absorbance at 280 nm in 50 mM sodium phosphate, pH 7.0, 1% (w/v) sodium dodecyl sulfate [23]. Suspensions of SR vesicles, 35–40 mg/ml, were stored at −70°C.

2.3. Measurement of maximum Ca^{2+} transport by SR vesicles

The maximum rates of Ca^{2+} transport were determined by the Fluo-3 method. SR vesicles, 0.25 mg/ml, were incubated under standardised conditions with 5 mM oxalate at 25°C, and 20 μM $CaCl_2$. ATP, 2 mM, was added and fluorescence was recorded. At 200 s a single pulse of either 20 or 100 μM Ca^{2+} was added to the cuvette and the time taken for fluorescence to reach $(F_{max}-F_{min})/2$, the peak width at half height, was recorded. This was assumed to be the time for the pulsed addition of Ca^{2+} to be transported into vesicles and to be stored as calcium oxalate. It was also assumed that for 95% of the timed period, the Ca^{2+} pump was operating at V_{max}, since the $K_{0.5}$ of Fluo-3 of 450 nM is equivalent to the $K_{0.5}$ of the Ca^{2+} transport system [2]. Typical widths of a 100 μM peak of fluorescence for uninhibited and 90% inhibited transport were 30 and 300 s, respectively.

2.4. Determination of steady state levels of extravesicular $[Ca^{2+}]$, Ca^{2+} uptake and release

The kinetics of calcium uptake and release, and of steady-state levels of extravesicular free $[Ca^{2+}]$ were monitored under standard conditions at 25°C in medium containing 20 mM MOPS/Tris, 20 mM histidine (pH 6.8), 5 mM $MgCl_2$, 5 mM sodium oxalate, and 20 nM Fluo-3. SR vesicles, 0.25 mg/ml, were used in all experiments reported here. Fluorescence was recorded in a 1-cm cuvette with continuous magnetic stirring, using a SPEX Fluoromax spectrofluorimeter, with excitation at 509 nm, emission at 535 nm, and both slit widths of 1 mm. Free $[Ca^{2+}]$, in nM, was calculated assuming K_ds for Ca^{2+} binding of 450 nM at 25°C [25], and of 864 nM at 37°C [26], according to the equation $[Ca^{2+}]_{free} = K_d \times (F-F_{min})/(F_{max}-F)$, where F is the observed fluorescence, and F_{max} and F_{min} are the fluorescence with 20 μM Ca^{2+} and 5 mM EGTA, respectively. A linear relationship between temperature and K_d was used to calculate free $[Ca^{2+}]$ with inter- and extrapolation in temperature dependence studies in the range 10–45°C.

2.5. Simulation of catalytic intermediates under steady-state conditions of the Ca^{2+}-limited state

Steady-state intermediates were determined by numerical integration of a 12-component unbranched catalytic cycle of the Ca^{2+}-ATPase, using rate constants given by Inesi and de Meis [27]. Simulation was begun with ligand-free enzyme and continued for 2 s in steps of 0.1 ms. Accuracy of the method was checked by summing all intermediates, which equaled that of total enzyme of 1.000 μM.

3. Results

3.1. Characterisation of SR vesicles at the Ca^{2+}-limited state

The kinetics of Ca^{2+} uptake and release by SR vesicles were studied, using the Ca^{2+} fluorophore, Fluo-3. The $K_{0.5}$ for this probe, of 450 nM at 25°C, is convenient since it matches the high-affinity Ca^{2+}-binding sites of the Ca^{2+}-ATPase, and its sensitivity is such that 20 nM of the probe gives a stable fluorescence signal with a good signal to noise ratio. Inclusion of 20 mM histidine in the medium minimises contribution of endogenous Zn^{2+} to total fluorescence [22]. Calculation of free $[Ca^{2+}]$ was based upon initial F_{max} and of F_{min}, following addition of 5 mM EGTA. Typically F_{min} was 3–4% of F_{max}. No significant effects of light scattering, due to formation of intravesicular calcium oxalate crystals, were encountered. From the increase in light emission fol-

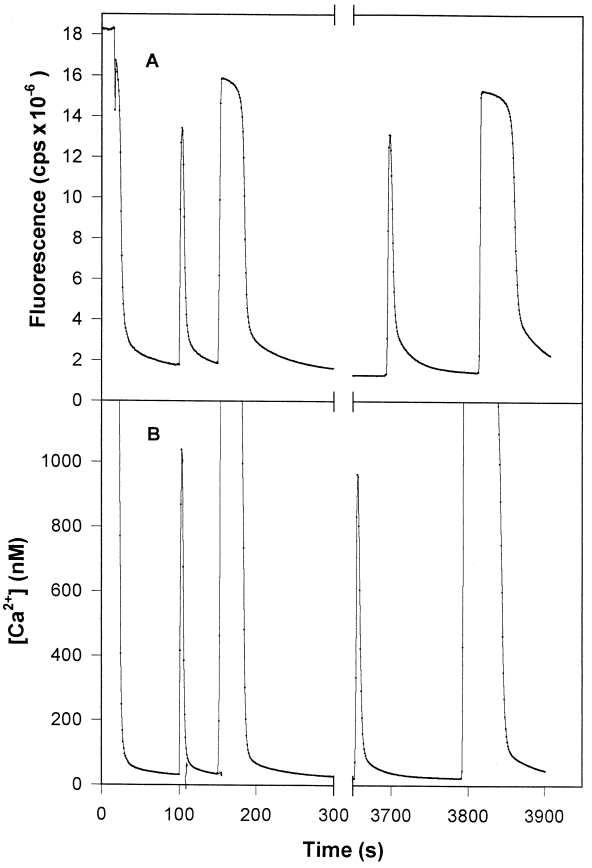


Fig. 1. Ca^{2+} uptake by SR vesicles and the Ca^{2+}-limited state. SR vesicles, 0.25 mg/ml, were incubated in standard medium containing 20 μM Ca^{2+} and 5 mM oxalate. ATP, 2 mM, was added at 20 s, following which pulses of 20 and 100 μM Ca^{2+} were added. Following incubation at 25°C for 1 h, additions of Ca^{2+} were repeated. The fluorescence trace of Fluo-3 is shown in A, and its transform to $[Ca^{2+}]_{free}$, according to the technique described in Section 2, is shown in B.

lowing active loading of up to 20-fold extra Ca^{2+} (2 μmol/mg), it could be calculated that the expected positive error from this source in a standard experiment was equivalent to 0.2 nM free Ca^{2+}.

Active transport by SR vesicles, reduces medium $[Ca^{2+}]_{free}$ to less than 0.1 μM in the presence of oxalate. A typical recording of fluorescence following addition of ATP and pulsed additions of Ca^{2+} is shown in Fig. 1A, and its transform to $[Ca^{2+}]_{free}$ in Fig. 1B. Ca^{2+}, 20 μM, was preincubated with SR to promote 'seeding' of calcium oxalate, on initiating Ca^{2+} transport with ATP to obviate supersaturation. The immediate sharp decrease in fluorescence signal is due to chelation of Ca^{2+} by ATP, since it occurred following preincubation with TG (data not shown). Limiting $[Ca^{2+}]$, or $[Ca^{2+}]_{lim}$, is typically in the range 45–54 nM [22]. The steady-state level of $[Ca^{2+}]_{lim}$ was unchanged for up to 2 h at 25°C, provided that ATP concentrations were maintained above the 0.1 mM level (data not shown). Efflux of Ca^{2+} followed substrate depletion at rates of 0.02% of V_{max} [22]. Intravesicular $[Ca^{2+}]$ in the presence of 5 mM oxalate has been estimated to be approximately 10 μM [28].

3.2. Effects of inhibitors of active transport by Ca^{2+}-ATPase on release of Ca^{2+} from SR vesicles at $[Ca^{2+}]_{lim}$

At the steady state of $[Ca^{2+}]_{lim}$ the sum of Ca^{2+} release, by means of several different efflux pathways, will equal active transport. Inhibitors of active transport by SR vesicles, had varying effects (Fig. 2A). Addition of vanadate was followed by a stable $[Ca^{2+}]_{lim}$ for up to 10 min. This would suggest that active transport is minimal at the steady state. The possibility that intravesicular Ca^{2+} could be precipitated as the Ca^{2+}–VO_4^{3-} complex would not be expected to affect interpretation of the data. The Ca^{2+} ionophore, A23187, released a large fraction of the Ca^{2+} that had been loaded into SR vesicles as calcium oxalate. The non-ionic detergent, Triton X-100, solubilised SR membranes and released intravesicular Ca^{2+} stores. Differences in the emitted fluorescence signal between A23187 and Triton may be explained by the effect of the detergent on light scattering from vesicles. The effects of A23187 and Triton show that release of free Ca^{2+} from calcium oxalate precipitates within the SR lumen is not a factor limiting efflux when Ca^{2+} uptake is inhibited.

The effects of thapsigargin (TG), a specific inhibitor of the Ca^{2+}-ATPase, were unexpected. Addition of 5 μM TG to 1 μM ATPase caused a slow release of Ca^{2+} from intact vesicles ($t_{0.5} = 150$ s), which amount was less than that with ionophore or detergent. It appears that TG released Ca^{2+} from a pool that is different from the bulk of Ca^{2+}, stored in the form of calcium oxalate. This is clearly shown in Fig. 2B, where A23187 was able to release Ca^{2+}, in addition to that released by TG. Vanadate had little effect on the amount of Ca^{2+} released by TG (Fig. 2C). It did, however, superimpose a small linear efflux, in excess of that from TG alone.

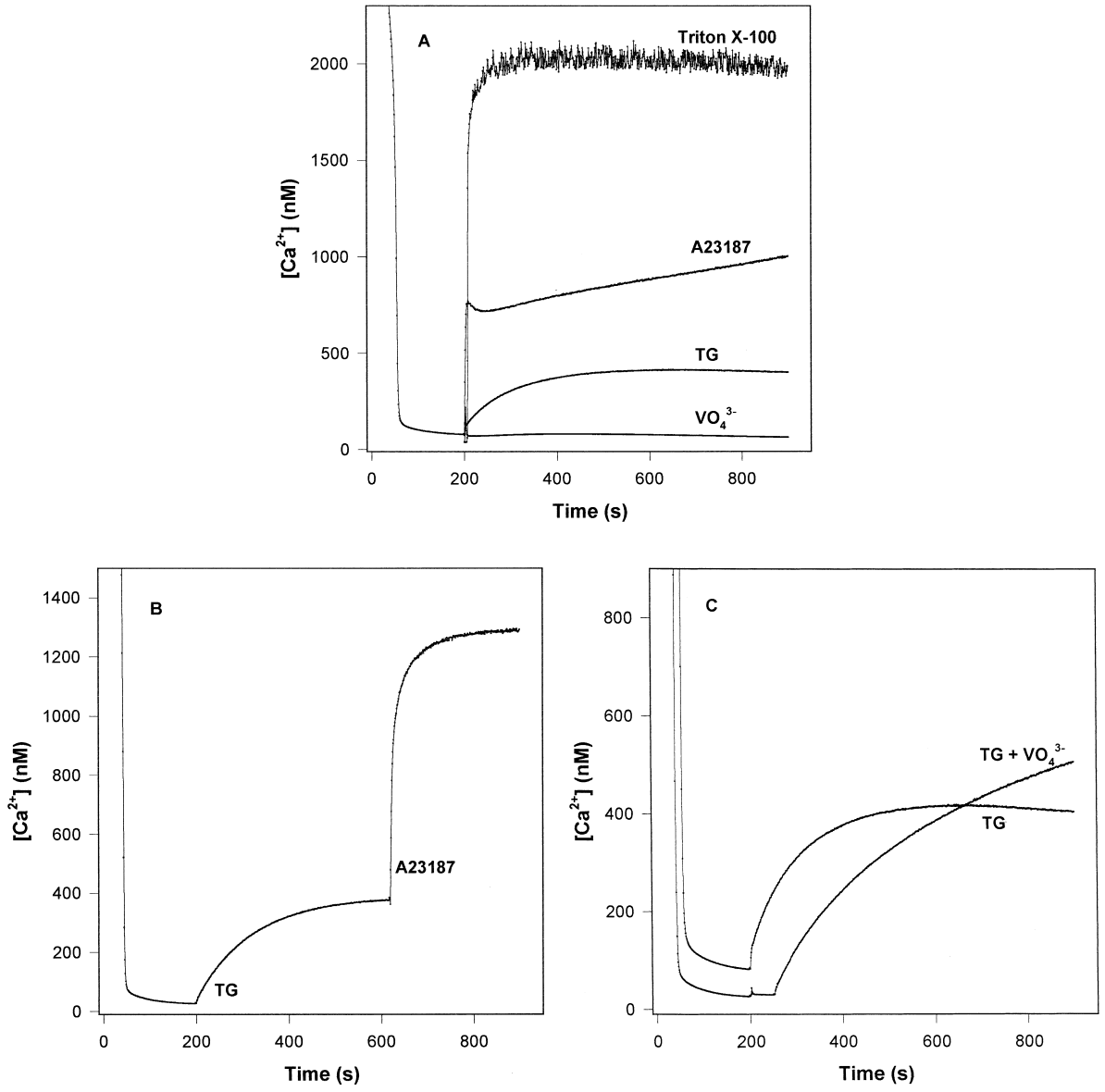


Fig. 2. Effect of TG and inhibitors of net Ca^{2+} transport by SR vesicles. In A, SR vesicles were incubated in standard medium, as described in Fig. 1. TG, 5 μM, vanadate, 1 mM, A23187, 2% (w/w), or Triton X-100, 1% (w/w), were added at 200 s. In B TG, 5 μM, was added at 200 s, and A23187, 2% (w/w), at 600 s. The effect of preincubation with 1 mM VO_4^{3-} added at 200 s, followed by 5 μM TG at 230 s, is shown in C.

3.3. *Stoichiometry of thapsigargin–Ca^{2+}-ATPase interaction for Ca^{2+} release*

Thapsigargin binds to the Ca^{2+}-ATPase, with a K_d of less than 10^{-9} M [29,30]. The [TG]-dependence of the maximum amount of Ca^{2+} released was shown to be non-linear (Fig. 3A). In the presence of 1 μM Ca^{2+}-ATPase (0.25 mg/ml), TG caused little effect below a 1:1 ratio. This then increased up to 2:1 ratio and reached a plateau at higher TG concentrations. In the range 1–5 μM TG the initial efflux reached a maximum and then appeared to undergo reuptake. The reuptake phase was inhibited in the 2–10 μM TG range. TG inhibition of transport showed a different stoichiometry (Fig. 3B). Inhibition was linear and maximal at 0.5:1 TG/ATPase. There are several uncertainties in establishing molar ratios of TG/Ca^{2+}-ATPase. The concentration of stock solutions

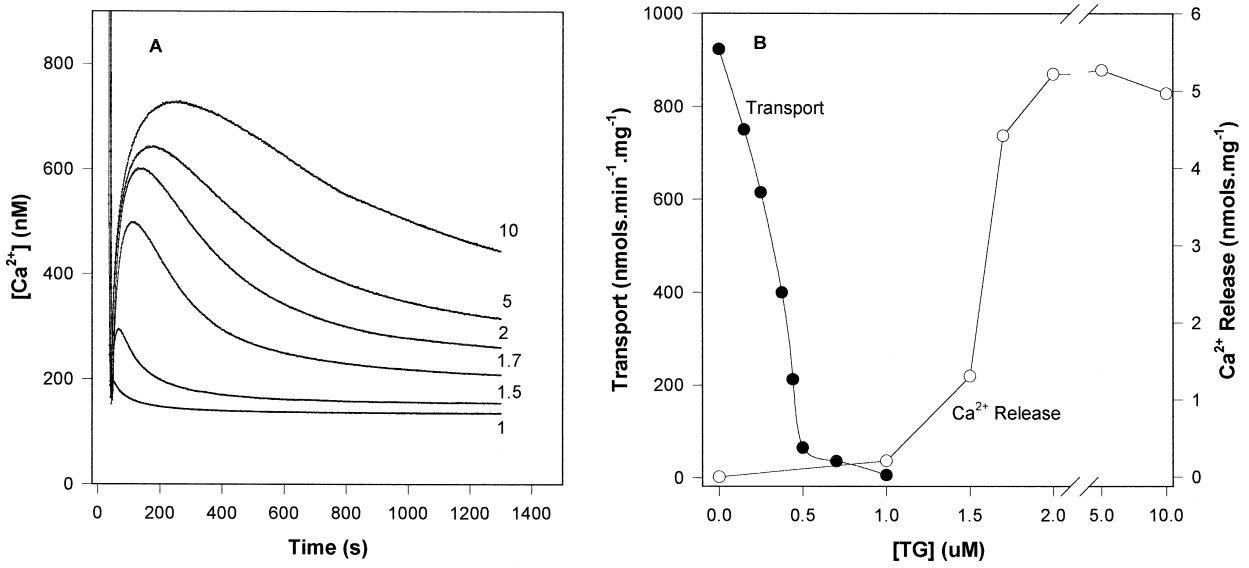


Fig. 3. Effects of varying TG on Ca^{2+} release, reuptake and on Ca^{2+} transport. In A, SR vesicles, 0.25 mg/ml, were preincubated in standard medium with 20 μM Ca^{2+} added. ATP, 2 mM, was added at $t=0$ s, and varying [TG], in μM alongside each curve. The amount of Ca^{2+} released was estimated from a biexponential fit to correct for the reuptake phase, and is shown in B. Inhibition of Ca^{2+} transport by TG was determined in separate experiments following preincubation for 1 min at varying amounts of TG. Transport was initiated with 2 mM ATP and determined from the pulse width at half height of a peak of Ca^{2+}, 100 μM, added at 200 s. Data are plotted in B.

of TG rely on Suppliers' data, and the compound is presumed to be stable during storage at 4°C. Of more concern are measurements of the enzyme itself. Maximum E-P levels of phosphorylation from $^{32}P_i$ are approx. 4.0 nmol/mg, which indicates that at least 50% of the SR preparation may be inactive.

3.4. Quantitation of Ca^{2+} released by thapsigargin

Determination of the amount of Ca^{2+} released into the medium by TG is dependent upon the change in $[Ca^{2+}]_{free}$, monitored by Fluo-3, and the buffering capacity of the medium for Ca^{2+}. Buffering was determined by addition of known aliquots of Ca^{2+} to the medium, once $[Ca^{2+}]_{free}$ had reached its maximum, following addition of TG (Fig. 4, inset). Ca^{2+} buffering, defined as the amount of added Ca^{2+} required to cause an equimolar increase in medium $[Ca^{2+}]_{free}$, was 2.62 in medium containing 5 mM oxalate, and 2.80 in 10 mM phosphate buffer (Fig. 4). In this experiment the maximum amount of Ca^{2+} release increased medium $[Ca^{2+}]_{free}$ by 375 nM, which allowing for Ca^{2+} buffering represents release of 3.9 nmol/mg or 0.98 mol/mol Ca^{2+}-ATPase.

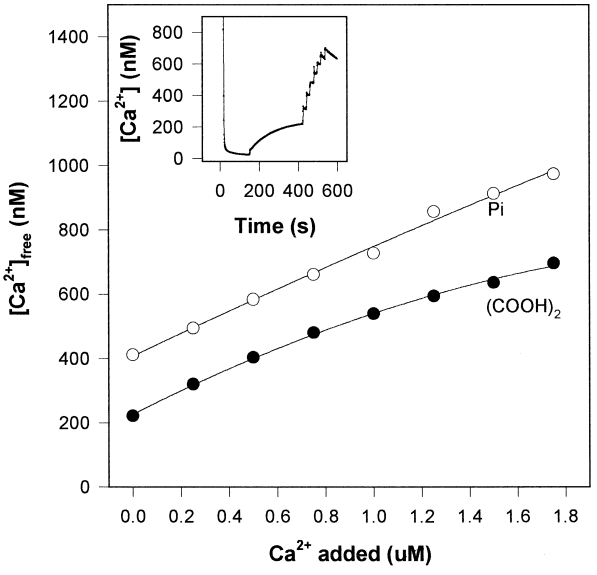


Fig. 4. Buffering of released Ca^{2+} by oxalate and phosphate. Under standard conditions with either 5 mM or 10 mM phosphate, ATP, 2 mM was added at 0 s, and TG, 5 μM, at 150 s. When the signal was steady at 400 s, $CaCl_2$ was added in 0.25-μM increments to a total of 1.75 μM (see inset). Buffering capacity for Ca^{2+} was calculated from the initial slope of a binomial fit of the data. Buffering capacity was 2.62 and 2.80 for oxalate and phosphate, respectively.

3.5. Variation in magnitude and kinetics of TG-releasable Ca^{2+} with interval following active transport

Preliminary experiments indicated that the characteristics of the TG-releasable Ca^{2+} fraction were dependent on the delay between activation of Ca^{2+} uptake by ATP and addition of TG. As expected, molar excess of TG added to the reaction medium prior to addition of ATP completely abolished the Ca^{2+} uptake phase. A series of experiments with increasing delay period from 20 to 1000 s is shown in Fig. 5A. TG, added 300–1000 s after the period of active transport, whilst in the steady state of $[Ca^{2+}]_{lim}$, showed a single monotonic phase of release, with rate constant $k \cong 0.01$ s^{-1}. For intermediate intervals, the kinetics of release were more complex, in that a relatively rapid phase of release caused an 'overshoot', following which there appeared to be a phase of reuptake or binding of Ca^{2+} to the SR. The final steady-state $[Ca^{2+}]_{free}$ for delay periods between 20 and 1000 s was in the range 250–300 nM. The amplitude of the initial rapid rate of Ca^{2+} release, shown in Fig. 5B, was 3.0 nmol/mg, declining to 0.9 nmol/mg at 1000 s. The rate constant for this decline of the rapid phase was 0.13 min^{-1}. Concomitantly the rate constant for release declined from an initial 0.11 s^{-1} at 20 s delay to approximately 0.0075 s^{-1} at 1000 s. The rate constant for decline in the rapidly effluxed Ca^{2+} at minimal delay times was 2.3 min^{-1}.

3.6. Effects of loading with alternate substrates

Acetyl phosphate (AcP) and GTP support Ca^{2+} uptake into SR vesicles, but there is no possibility of a fully reversible pump cycle. Fig. 6A shows that release of Ca^{2+} by TG is slower but greater with vesicles that are loaded in the presence of AcP or GTP. However, the release phase is modified in vesicles that are actively loaded by GTP (Fig. 6B). Here release is slower and there is no reuptake phase as seen with ATP (cf. Fig. 5A). Differences in the kinetics of efflux and reuptake phases between ATP and alternate substrates may be relevant for the mechanism of reuptake (see Section 4).

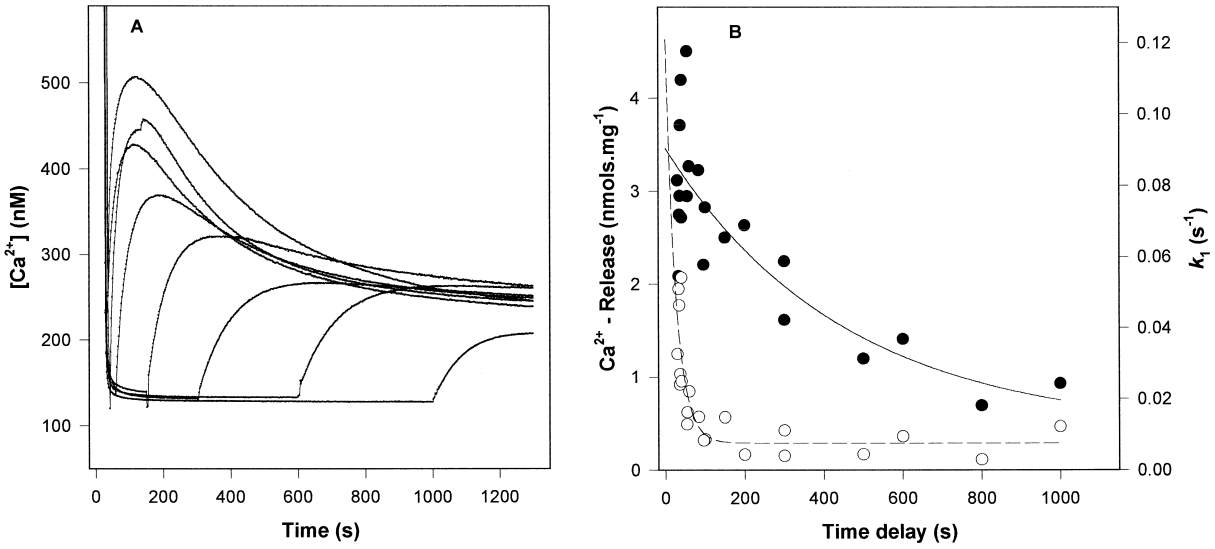


Fig. 5. Effects of a delay between onset of transport and addition of TG. In A, under standard conditions, ATP was added at 10 s, and 5 μM TG at varying intervals up to 1000 s. The amount of Ca^{2+} released by TG was determined from a biexponential fit of the curves up to 300 s delay. Longer delay curves were fitted to a single exponential. The data for Ca^{2+} release (filled circles) and rate constants, k_1 (open circles) shown in B, which include results of several additional experiments, were fitted to a single exponential decay. The best fit of Ca^{2+} released (solid line) had an amplitude of 3.04 nmol/mg ATPase, and decay constant, k, of 0.13 min^{-1}. The first-order rate constants for Ca^{2+} release versus delay were also fitted to a single exponential decay curve (dashed line)..

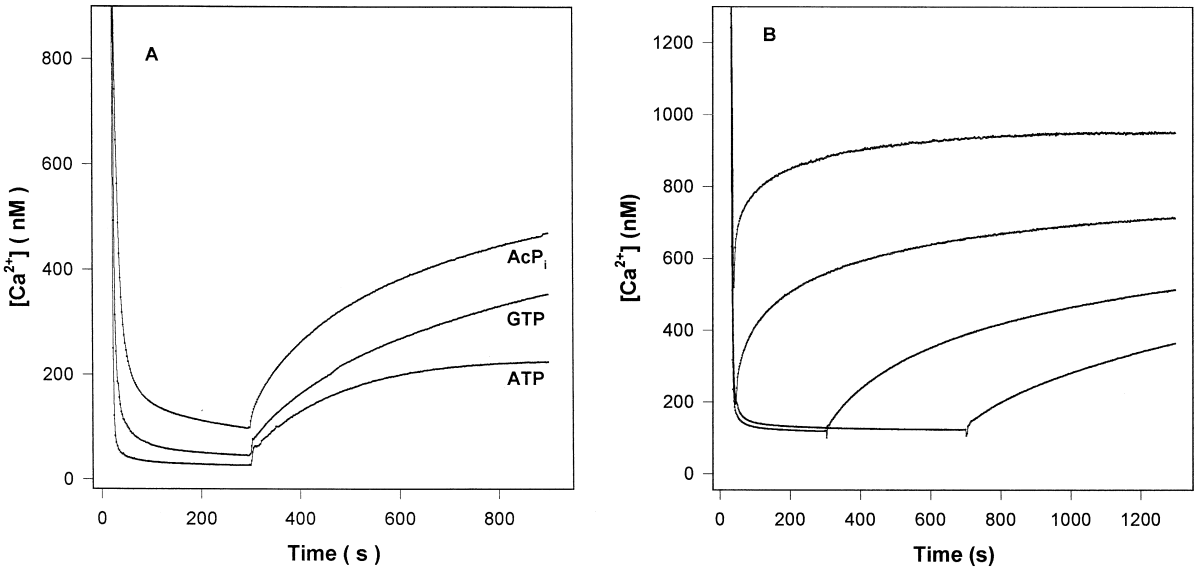


Fig. 6. Effects of pseudosubstrates on TG-induced Ca^{2+} release. ATP, 2 mM, GTP, 2 mM, and AcP, 10 mM, were added to SR vesicles, as before, and TG, 5 μM, at 300 s and $[Ca^{2+}]_{free}$ monitored in A. Effects of varying delay between onset of transport with 2 mM GTP and addition of 5 μM TG are shown in B.

3.7. *ADP-sensitivity of thapsigargin-induced Ca^{2+} release*

Specific release of Ca^{2+} by TG indicates that its mechanism of action must involve its interaction with the Ca^{2+}-ATPase. In that case release of a fraction of total Ca^{2+} taken up into SR vesicles may be explained by reversal of a single pump cycle with release of Ca^{2+}. Reversal of the cycle would include binding of ADP. ADP is expected to be present in the medium under the usual experimental conditions, assuming 10% hydrolysis of substrate, which would release 200 μM ADP. Addition of phosphoenol pyruvate and pyruvate kinase, which should convert all ADP present to ATP, had no effect on the extent of TG-released Ca^{2+}. Likewise addition of ADP, up to 1 mM, did not facilitate Ca^{2+} release (data not shown).

3.8. *Variable loading of SR vesicles with Ca-oxalate and its effect on Ca^{2+} release*

It is possible that during active transport lumenal $[Ca^{2+}]_{free}$ increases above 10 μM, the expected equilibrium value. In that case, Ca^{2+} release would be expected to vary with the degree of loading of SR vesicles if TG-induced Ca^{2+} release was through a release channel, or by passive flow of Ca^{2+} through an ionophoric pathway. In a series of experiments Ca^{2+} loading was varied from 0 to 2000 nmol/mg SR and effects of 5 μM TG measured at 300 s delay. Active loading of up to 800 nmol/mg had no effect on the amount of Ca^{2+} released by TG, which was 3.4 ± 0.23 nmol/mg and 1.7% of the amount of Ca^{2+} actively transported. With 2000 nmol/mg of Ca^{2+}, $[Ca^{2+}]_{lim}$ was elevated and virtually all of the Ca^{2+} loaded was released by TG. Since most assays of release were with 80 nmol/mg Ca^{2+} it follows that altered loading capacity did not influence the amounts of Ca^{2+} released by TG.

The specific inhibitor of the Ca^{2+} release channel, Ruthenium red, 2 μM, had no effect on Ca^{2+} release (data not shown), indicating that the physiological ryanodine-sensitive release channel does not contribute to TG-induced release.

4. Discussion

Isolated sarcoplasmic reticulum (SR) vesicles, prepared from rabbit skeletal muscle, provides near ideal conditions for studies on active transport in P-type cation pumps. Vesicles are well sealed and the Ca^{2+}-ATPase pump protein is approximately

85% pure. Pump units are physiologically orientated, with external ATP-binding sites representing the in vivo situation, where out is synonymous with the cytosolic surface [1]. Active transport has been studied both by means of chelatometric dyes, either by absorbance or fluorescence, or by measurements of fluxes of $^{45}Ca^{2+}$ into or out of vesicles following filtration to separate vesicles from medium. Such studies are generally performed in the presence of Ca^{2+} chelators, either the reporting dyes themselves, or with CaEGTA buffers to control free [Ca^{2+}] in the submicromolar range, which is relevant for physiological studies. It is also in the range where activation of many Ca^{2+}-dependent signalling events occur. A feature of the present study is that it depends on sequestration of contaminating medium Ca^{2+} into isolated SR vesicles to deplete medium Ca^{2+} in the absence of Ca^{2+} buffers.

Calcium uptake into SR vesicles in the presence of oxalate or phosphate anions decreases medium free Ca^{2+} to a level of approximately 50 nM, following which these levels are maintained for several hours at 25°C. Efflux pathways, which are minimal (0.02% of V_{max}), balance active uptake. This phenomenon has been termed $[Ca^{2+}]_{lim}$ to indicate that the Ca^{2+}-ATPase pump cycle is limited due to low medium (cytosolic) $[Ca^{2+}]_{free}$ [22]. Only a relatively small fraction of total Ca^{2+}, stored as Ca^{2+}oxalate inside vesicles, was released by TG. This is indicated in Fig. 2A, where ionophore A23187, and the non-ionic detergent, Triton X-100, caused rapid release of Ca^{2+}. Ionophore also increased medium Ca^{2+} once maximum effects of TG had occurred (Fig. 2B). The ultimate aim of this study was to establish the origins of the TG-releasable Ca^{2+} fraction.

The extent and kinetics of Ca^{2+} release and reuptake were dependent on a number of factors. [TG] dependence of active transport was linear at lower concentration, compatible with high-affinity titration of binding sites (Fig. 3B) [30]. There is, however, a difference in the apparent number of binding sites for TG upon the ATPase. Inhibition of transport was complete at 0.5 μM TG, whilst release was maximal at 2.0 μM TG. The concentration of enzyme, 0.25 mg/ml, represents 1.0 μM pump units. According to protein measurements, pure active enzyme should contain 8 nmol/mg of phosphorylatable EP sites. Measured maximum EP levels from P_i in the presence of EGTA are in the range 3.5–4.5 nmol/mg [31]. It has been suggested that 50% of Ca^{2+}ATPase protein is in the inactive form [32]. This readily explains the 0.5:1.0 ratio of TG to enzyme sites titrated in the transport inhibition experiments. The reason for the 2:1 titration of Ca^{2+} release sites is not obvious. The [TG]-dependence of Ca^{2+} release is not linear in the 0–2.0 μM range. Little Ca^{2+} is released up to 1.0 μM TG, but release increases sharply between 1.0 and 2.0 μM of inhibitor. A possible explanation is that in the lower range TG binds to both active and inactive pump units. A stoichiometry of 2:1 does, however, imply that at least some of the pump units that are inactive with regard to transport, nevertheless can bind TG. Residual active pumps, e.g., at 1 μM TG, may be capable of reuptake of Ca^{2+} released into the medium from TG-liganded enzyme.

Thapsigargin binds rapidly and tightly to the Ca^{2+}-ATPase with K_d of 0.2 nM [33] to form a 1:1 complex. The inhibited enzyme shows decreased affinity and binding of Ca^{2+} that has been explained by preferential binding to the E_2 conformation, with irreversible formation of the dead-end complex, E_2^A.TG [34,35]. From these observations it was concluded that the inhibitor does not bind to species other than the unliganded E_2. However, failure of molar ratios of more than 1:1 for TG/E to further decrease Ca^{2+} affinity, and effects of the related inhibitor, thapsivillosum A, on the kinetics of quenching of tryptophan, and their reversal by Ca^{2+}, lead Wictome et al. [17] to conclude that the sesquiterpene lactones bind with equal affinity to E_1Ca_2, E_1 and E_2. The observed decrease in affinity for Ca^{2+} is due to a high equilibrium constant of 5.0×10^3 for E_2^ATG/E_2.TG to give an overall binding constant for $E_2+TG\leftrightarrow E_2^ATG$ of 4.0×10^{10}, where E_2^ATG is the stable isomer of E_2TG [17]. It follows that in the experiments reported here, addition of a molar excess of TG to SR vesicles in the Ca^{2+}-limited steady state would result in rapid inhibition (<2 s) of the pump with virtually all of enzyme sequestered as the E_2^ATG complex. Both proposed reaction schemes predict that in the presence of excess TG greater than 99% of the total enzyme will be in the Ca^{2+}-free E_2 form.

A reaction scheme for the Ca^{2+}-ATPase pump cycle has been formulated by Inesi and De Meis [36] that includes 12 intermediate states and their

associated first- and second-order rate constants at 25°C. It is assumed in the present study that $[Ca^{2+}]_{out}$ is 100 nM, $[Ca^{2+}]_{in}$ is 10 μM, and [ATP], [ADP] and $[P_i]$ are 1.8, 0.2 and 0.2 mM, as a result of 10% hydrolysis of the initial 2 mM ATP. Steady-state levels of all intermediate species were determined by numerical integration, and the results are shown in Table 1. Total phosphorylated species is 0.27%, and total Ca^{2+} bound species 0.93%. It is possible that lumenal $[Ca^{2+}]_{free}$ is in excess of the value, 10 μM, of the equilibrium value for calcium oxalate precipitation. A simulation for intralumenal $[Ca^{2+}]_{free}$ of 1 mM has also been calculated, in which case total E-P and total Ca^{2+}-bound species would be 0.75% and 1.64%, respectively.

Assuming that a major fraction of the Ca^{2+}-ATPase at $[Ca^{2+}]_{lim}$ is in the Ca^{2+}-free form, and that simulation predicts that TG will convert all intermediates into a form that excludes Ca^{2+} binding, it can be assumed that the phenomenon of TG-induced Ca^{2+} release cannot be explained on the basis of the reaction mechanisms quoted above.

Several properties of Ca^{2+} release suggest that the Ca^{2+}-ATPase is the target for TG related effects. Stoichiometry of TG/E of 2:1 is compatible. Stoichiometry of Ca^{2+} released varies between 0.25 and 1.0 Ca^{2+}/mol ATPase. Our preparation of SR vesicles is enriched with cisternal SR, from which terminal release channels are excluded. Ruthenium red, which blocks ryanodine sensitive Ca^{2+} release channels, had little effect on the amount and kinetics of TG-induced Ca^{2+} release.

Inesi and De Meis [36] have characterised Ca^{2+} fluxes across SR vesicles in the absence of oxalate, where Ca^{2+} uptake is inhibited by accumulation of millimolar intravesicular Ca^{2+}. Under these conditions the net flux ceases due to inhibition of Ca^{2+} release from E_2-P.2Ca. Passive Ca^{2+} efflux in this steady state is equally divided into efflux through the lipid bilayer, slippage of the Ca^{2+} pump, and via cycling of Ca^{2+} bound $E_2 \leftrightarrow E_1$ forms in the absence of phosphorylation.

Reuptake of Ca^{2+} following the release phase varied. It was maximal immediately after ATP addition and declined later until undetectable at 5 min delay. It was also highly pH dependent, being absent at pH 6.0, and maximal at pH 8.0, where almost all of the Ca^{2+} released was taken up within 2 min (data not shown). Reuptake was decreased, but not abolished by TG in the 2–10 μM range, and was almost com-

Table 1
Simulation of steady-state concentrations of intermediate species of the Ca^{2+}-ATPase catalytic cycle in the Ca^{2+}-limited state

Intermediate	$[Ca^{2+}]_{in}$ = 10 μM		$[Ca^{2+}]_{in}$ = 1 mM	
	(M)	(%)	(M)	(%)
E_1	7.62 E-09		8.97 E-07	
$Ca.E_1$	3.80 E-09		4.39 E-09	
$Ca.E_1$*	1.56 E-09		1.80 E-09	
$Ca2.E_1$*	1.07 E-11		1.58 E-11	
$Ca2.E_1$*.ATP	7.44 E-10		1.32 E-09	
$(Ca)2.E_1$*∼P.ADP	1.79 E-10		3.62 E-10	
$Ca2.E_2$*–P.ADP	1.72 E-10		3.93 E-10	
$Ca2.E_2$*–P	9.07 E-11		5.53 E-10	
$Ca.E_2$*–P	1.38 E-09		3.16 E-09	
$Ca.E_2$–P	1.38 E-09		1.73 E-09	
E2–P	1.15 E-07		5.37 E-08	
$E2.P_i$	1.15 E-07		5.31 E-08	
Total intermediate species	1.000 E-06	(100)	1.000 E-06	(100)
Total E-P species	2.70 E-09	(0.04)	7.52 E-09	(0.75)
Total bound Ca^{2+}	9.27 E-09	(0.93)	1.64 E-08	(1.64)

Concentrations of reaction intermediates were simulated from a reaction scheme and rate constants according to Inesi and de Meis [36], which were originally determined under conditions of T = 25°C, 5 mM $MgCl_2$, pH 7.0 and 80 mM KCl. Initial conditions were set at ATP = 1.8 mM, ADP = 0.2 mM, P_i = 0.2 mM, $[Ca^{2+}]_{free}$ = 50 nM and $[E]_{total}$ = 1.0 μM. Simulation was carried out by numerical integration, with a step interval of 0.1 ms, and total integration time of 2 s.

pletely inhibited by 1 mM VO_4^{3-}. Taken together these findings suggest that Ca^{2+} reuptake is due to slow turnover of the Ca^{2+} pump in the presence of TG. The reason why reuptake is not seen with GTP or AcP as substrates is not clear (Fig. 6). One possibility is that it may be related to the specificity of the 'regulatory' nucleotide site that accelerates several of the intermediary reactions involved in the formation and hydrolysis of E_2-P, and which have a high specificity for ATP.

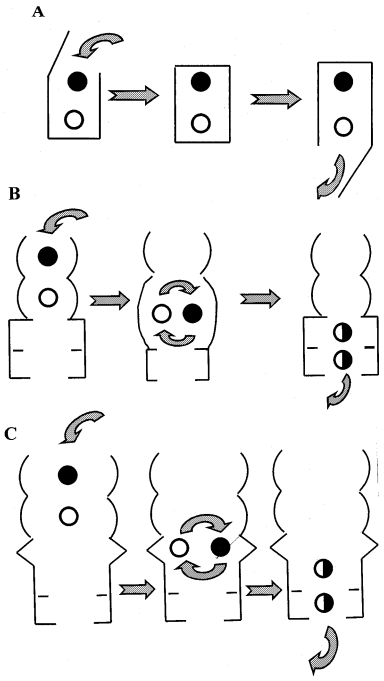
The alternating access model for the $E_1 \leftrightarrow E_2$ isomerisation step initially was viewed as a pair of Ca^{2+} sites that switched orientation and affinity with phosphorylation and dephosphorylation of the ATPase [37,38]. Positive cooperative binding to two sites within a channel-like structure has been proposed, based on the inhibition of release of the deeper site, labelled with $^{45}Ca^{2+}$, by medium $^{40}Ca^{2+}$ [4,6,33,39]. Transfer of two Ca^{2+} to lumenal sites on phosphorylation is followed by dephosphorylation and randomised release to the lumen [35,40]. Forge et al. [12] and Duggleby et al. [41] have described biphasic release of Ca^{2+} from lumenal sites at pH 8.0 and 5°C, suggesting that release of Ca^{2+} from low-affinity sites is also sequential. A single channel-like mechanism predicts that the deeper cytosolic site would be released to the lumen earlier than the more superficial site. However, the rate of release of $^{45}Ca^{2+}$ was similar when label was bound either to deep or superficial cytosolic sites. Together, these data show that although both E_1 and E_2 sites bind and release sequentially, the two transported Ca^{2+} ions are randomised between these intermediate states.

Occluded cation species are believed to be a common feature of all P-type cation pumps [42]. The occluded E-P⟨2Ca⟩ form, representing the intermediate state between E_1-P.2Ca and E_2-P.2Ca, is transient and has not been observed directly under steady-state conditions, since E-P.2Ca is intrinsically unstable. It only deoccludes by a slow conformational change or by reverse dephosphorylation by ADP. It is, however, formed rapidly and is stable for hours when a non-covalent 'EP-like' state is formed with CrATP [43]. Chemical modification of the ATPase at its active site also results in stabilising the occluded Ca^{2+} intermediate. McIntosh et al. have shown that glutaraldehyde inhibits enzyme activity by forming an intramolecular cross-link at the active site between Lys^{492} and Arg^{678}, leading to a prolonged phase of the occluded Ca^{2+} species when phosphorylated by acetyl phosphate [44]. In this case deocclusion via the low-affinity Ca^{2+} sites to the lumen is blocked. Instead, occluded Ca^{2+} is released to the cytoplasmic surface, through a pathway that uncouples the transport cycle [45].

The fact that the stoichiometry of the TG-released Ca^{2+} fraction has a maximum of 3.0–4.0 nmol/mg ATPase, equivalent to 0.75–1.0 mol/mol ATPase, is perhaps the strongest evidence for an occluded species E⟨Ca^{2+}⟩, that accumulates during enzyme turnover, and which slowly decays during the Ca^{2+}-limited state. A species of tightly bound Ca^{2+} has been identified on the SR Ca^{2+}ATPase that is not removed at lowered temperature with EGTA, but can be released under conditions, including mild acid, and EGTA at 37°C, that uncouple transport from ATPase activity [46]. Ca^{2+}, in addition to that binding to transport sites, has been shown to be necessary for full activation of the Ca^{2+}-ATPase [47]. Prior incubation with EGTA leads to inhibition of activity, which is partially restored by preincubation with Ca^{2+}. Plasma membrane Ca^{2+}-ATPase also contains a species of Ca^{2+} that is firmly bound, not chased by EGTA, and independent of the transport sites, that has been suggested to play a structural role [48].

The present study has identified a species of Ca^{2+} that can be released from the Ca^{2+}-ATPase by TG under conditions that favour vacant cytoplasmic and lumenal Ca^{2+} binding sites. Simulation indicates that unliganded E_1 and E_2 account for $>99\%$ of the total enzyme. One possibility is that in the presence of TG the enzyme acts as an ionophore for Ca^{2+}, via cycling of the non-phosphorylated forms E_1 and E_2. Channel-like behaviour is unlike the fixed stoichiometry of partial release of intravesicular contents of calcium oxalate. Another more favoured hypothesis is that during the Ca^{2+}limited state, when $[Ca^{2+}]_{free}$ is less than 100 nM, occluded Ca^{2+} persists and is stabilised for lengthy periods of up to 1–2 h.

Possible models of active Ca^{2+} transport are shown in Scheme 1. The E_1–E_2 model, as originally described, included a pair of sites with two major conformational states with high and low affinities, controlled by gates, which allowed alternate access to the two membrane surfaces, and shown in Scheme 1A. However, no single large conformational change



Scheme 1. Possible models of active Ca^{2+} transport.

has been found, rather a number of smaller conformational changes have been followed by tryptophan autofluorescence on binding and release of ligands to the Ca^{2+}-ATPase. Alternatively, there is evidence for a four-site model.

Mezaros and Bak [7] showed that it is possible to simultaneously bind Ca^{2+} to E_1 and occlude bound Ca^{2+}. Jencks et al. [8] described how lumenal Ca^{2+} inhibits phosphorylation from P_i due to coexistence of both high-affinity cytoplasmic and low-affinity lumenal sites on the non-phosphorylated ATPase. Canet et al. [12] also showed that although Ca^{2+} release from cytoplasmic and lumenal sites are sequential, suggesting that they are bound in some sort of channel, their transfer results in randomisation. Consequentially they have suggested that ligand bonds are weakened in the occluded state to explain this randomisation (Scheme 1B). The present study, which demonstrates coexistence of the occluded state as well as low and high-affinity sites supports a mechanism with randomisation in the occluded species (Scheme 1C). This differs from Scheme 1B in that here randomisation involves the deeper site of two each of the of the high- and low-affinity sites, whereas in mechanism C occluded sites exist independently.

The standard E_1–E_2 reaction scheme includes two Ca^{2+} binding sites per ATPase at any one instant. The present findings favour an occluded species in addition to the four-site models. The experimental system should provide opportunity to analyse occluded states, especially since the species is stable for hours at room temperature.

According to the present in vitro analysis, interaction of TG with SR or ER Ca^{2+}-ATPases, under conditions that approximate those in relaxed muscle, or in other tissues during the unstimulated state, would release 4 nmol Ca^{2+} per mg from sites on the ATPase. Some idea of the magnitude from this source can be calculated for cardiac muscle, where the content of SR in intact muscle tissue has been determined by Thapsigargin titration to be 8.0 mg/g [49]. Assuming that cellular water is approximately 70% (v/w), this would lead to an increase in total cytosolic Ca^{2+}, both free and bound, of 46 μM. The amount of Ca^{2+} released by direct interaction of TG with SERCA's needs to be considered when analysing the effects of this inhibitor on cellular Ca^{2+} metabolism.

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